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IL-22 and IL-22 Binding Protein (IL-22BP) Regulate Fibrosis and Cirrhosis in Hepatitis C Virus and Schistosome Infections

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Interleukin (IL)-22 acts on epithelia, hepatocytes, and pancreatic cells and stimulates innate immunity, tissue protection, and repair. IL-22 may also cause inflammation and abnormal cell proliferation. The binding of IL-22 to its receptor is competed by IL-22 binding protein (IL-22BP), which may limit the deleterious effects of IL-22. The role of IL-22 and IL-22BP in chronic liver diseases is unknown. We addressed this question in individuals chronically infected with schistosomes or hepatitis C virus (HCV). We first demonstrate that schistosome eggs stimulate production of IL-22 transcripts and inhibit accumulation of IL22-BP transcripts in schistosome-infected mice, and that schistosome eggs selectively stimulate production of IL-22 in cultures of blood leukocytes from individuals chronically infected with *Schistosoma japonicum*. High IL-22 levels in cultures correlated with protection against hepatic fibrosis and portal hypertension. To test further the implication of IL-22/IL-22BP in hepatic disease, we analyzed common genetic variants of IL22RA2, which encodes IL-22BP, and found that the genotypes, AA, GG of rs6570136 ($P = 0.003$; odds ratio [OR] = 2), and CC, TT of rs2064501 ($P = 0.01$; OR = 2), were associated with severe fibrosis in Chinese infected with *S. japonicum*. We confirmed this result in Sudanese (rs6570136 GG [$P = 0.0007$; OR = 8.2], rs2064501 TT [$P = 0.02$; OR = 3.1]), and Brazilians (rs6570136 GG [$P = 0.003$; OR = 26], rs2064501 TC, TT ($P = 0.03$; OR = 11]) infected with *S. mansoni*. The aggravating genotypes were associated with high IL22RA2 transcripts levels. Furthermore, these same variants were also associated with HCV-induced fibrosis and cirrhosis (rs6570136 GG, GA [$P = 0.007$; OR = 1.7], rs2064501 TT, TC ($P = 0.004$; OR = 2.4]). *Conclusions*: These results provide strong evidence that IL-22 protects against and IL-22BP aggravates liver fibrosis and cirrhosis in humans with chronic liver infections. Thus, pharmacological modulation of IL-22 BP may be an effective strategy to limit cirrhosis. (HEPATOLOGY 2015;61:1321-1331)

Abbreviations: CentF, central hepatic fibrosis; CTGF, connective tissue growth factor; HBV, hepatitis B virus; HCV, hepatitis C virus; HF, hepatic fibrosis; IL, interleukin; IL-22BP, IL-22 binding protein; ILCs, innate lymphoid cells; NetF, network fibrosis; LD, linkage disequilibrium; LPS, lipopolysaccharide; mRNA, messenger RNA; OR, odds ratio; PBMCs, peripheral blood mononuclear cells; PH, portal hypertension; SNPs, single-nucleotide polymorphisms; Th, T helper.

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Interleukin (IL)-22 is produced by a variety of hematopoietic cells, such as innate lymphoid cells (ILCs), lymphoid tissue-inducers,[1] T-helper (Th) cells,[2] and γ/δ T cells.[3] However, unlike most cytokines, IL-22 does not act on hematopoietic cells, but affects epithelia, hepatocytes, and pancreatic cells,[4,5] suggesting an important role for this cytokine at epithelial barriers of the intestine, skin, and lungs and in the liver and pancreas. Indeed, IL-22 stimulates innate immunity by promoting production of antimicrobial peptides,[2,6,7] secretion of mucus, and release of chemokines[8-10] and by enhancing cell mobility.[6,7,11] IL-22 also protects tissues from damage and mediates tissue repair.[5,12,13] Deregulated IL-22 responses may cause pathological inflammation,[12] abnormal cell proliferation,[14] and enhanced chemokine production as occurs in experimental models of psoriaris,[8-10] *Toxoplasma gondii*–induced ileitis,[15] and arthritis.[16] Furthermore, release of both IL-17A and IL-22 in the same inflammatory sites may aggravate pathology given that IL-17A enhances the proinflammatory effects of IL-22.[9,16,17] Few studies have evaluated the role of IL22/IL-22 binding protein (IL-22BP) in disease, and with the exception of skin psoriasis, it is not known whether this cytokine protects against or aggravates various human diseases. Here, we evaluated whether IL-22 and its inhibitor, IL-22BP,[18,19] influence liver disease in humans infected with hepatitis C virus (HCV) or schistosomes. Hepatic fibrosis (HF) and cirrhosis develop during chronic liver inflammation caused by HCV, hepatitis B virus (HBV), schistosomes, steatosis, and alcohol. Fibrosis is an excessive deposition of extracellular matrix proteins in healing lesions. HF and cirrhosis cause varices, ascites, liver failure, and death in millions of patients. IL-22 protects against acute hepatitis[20] and stimulates tissue regeneration[21] in experimental models of liver disease, but aggravates inflammation in a mouse model of HBV infection.[17] Furthermore, in a murine model of schistosomiasis, IL-22 was not detected during infection.[22] Intestinal ILC produce IL-22 in inflammatory conditions and when damage occurs to the intestinal barrier; therefore, we hypothesized that IL-22 is produced when schistosome eggs perforate the intestine. The consequences of these egg-induced intestinal lesions on the human immune response during schistosomiasis deserve further investigations given that most studies have focused on splenomegaly and on periportal fibrosis. Here, we present evidence that IL-22/IL-22BP play significant roles in HF and cirrhosis in patients with chronic schistosome and HCV infections.

Materials and Methods

Study Population and Evaluation of Hepatic Fibrosis. The human protocols were approved by the research ethics committees of the University of Gezira and the National Cancer Institute (Gezira, Sudan), the Hunan Institute of Parasitic Diseases (Yueyang, China), the University do Triangulo Mineiro (Uberaba, Brazil), Fiocruz (Salvador) and University Estadual de Pernambucco (Recife). The protocols were also approved by French INSERM ethics committees and by the CNIL.

All study samples are described in Table 1. Subjects infected with schistosomes were obtained from populations exposed to infection with *S. japonicum* (China) or *S. mansoni* (Brazil, Sudan) as described previously.[23,24] Individuals infected with HCV (genotypes 1, 2 or 3) were recruited among patients attending the outpatient clinic at the Instituto do Figado in Recife and at the Hospital das clinicas in Salvador (Brazil).

Evaluation of Hepatic Fibrosis. Fibrosis in individuals infected with schistosomes was evaluated with ultrasound (US) that was carried out with a portable ultrasound machine and a 3.5MHz convex probe (LOGIQ Book XP 2410786, Jiangsu, China), based on the standardized procedures of the World Health Organization (WHO).[25] Hepatic fibrosis in individuals infected with *S. mansoni* or *S. Japonicum* manifests as central fibrosis (CentF) around the central vein, and in *S. japonicum* infections, a network fibrosis pattern (NetF) in the parenchyma is also present. WHO guidelines graded CentF as A, B, C, CL, D, E, or F and NetF as GN if the diameter of the network mesh was less than 12mm (resembling fish scales) or GW if it is more than 12mm (resembling a

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Potential conflict of interest: Prof. Dessein consults for, owns stock in, and holds intellectual property rights with GENEPRED Biotechnologies. Dr. Bourliere consults for, advises, is on the speakers' bureau of, and received grants from Janssen, MSD, and Bristol-Myers Squibb. He consults for, advises, and received grants from Gilead. He consults for and advises AbbVie and Roche.

Table 1. Description of the Study Samples

	Pathogen	N Total (Cases/Controls)	Age (Mean) Case/Controls Exposure (Mean) Case/Controls	Women % Case/% Controls	Severe Fibrosis	Mild or No Fibrosis
Chinese	*S. japonicum*	327 (122/205)	Age: 44.1 yrs/52.2 yrs F. yrs: 25.5 yrs/34.8 yrs	13.1/26.0	CL^H, D, E, F with GN^H CL^M with GN^M or GN^H	A, B, C, CL^L with GN0
Sudan	*S. mansoni*	217 (68/149)	Age = exposure 47.1 yrs/50.7 yrs	17.2/28.7	CL, D, E, F with gall bladder wall thickening	A, B, C no gall bladder thickening
Brazil (1)	*S.mansoni*	186 (45/141)	Age: 46.6 yrs/40 yrs	47.8/62.9	CL, D, E, F	A, B, C
Brazil (2)	HCV	532 (210/322) 364 (210/154)	F3-F4/F0-F1/F0: Age: 57 yrs/52.1 yrs/53.7 yrs	57.0/53.8/52.9	F3+F4 (cirrhosis)	F0+F1+F2 or F0+F1
Brazil (3)	HCV	149 (53/96)	F3-F4/F0-F1/F0: Age: 57 yrs/52.1 yrs/53.7 yrs	39/46.2	F3+F4 (cirrhosis)	F0+F1+F2

Exposure to infection was evaluated with "years of fishing" (F. yrs) in Chinese fishermen and with age in Sudanese and Brazilians infected with *S. mansoni*. Duration of infection could not be accurately evaluated in HCV-infected individuals. The analysis presented in Table 4 (Cohort Brazil [2]) compares F0, F1, and F2 with F3 and F4. The analysis presented in Fig. 4B,C (Cohort Brazil [2]) compares F0 and F1 with F3 and F4.

tortoise shell). CentF A pattern is a normal liver struc A, diffuse echogenic foci or "starry sky" is graded CentF B, an uninterrupted thickness of the venous wall distinguished grade CL from grade C, a patchy pattern was graded as D, E, and as F if around portal veins.

WHO grading was modified for *S. japonicum* infections as described in Supporting Materials.

Fibrosis stages in HCV infections were determined on liver biopsies with the Metavir scale. All covariates (alcohol consumption, schistosome infections, mode of contamination, virus genotypes) were assessed either by interview or by genotyping. None of the HCV-infected individuals had active HBV or HIV infections.

Samples for immunological analysis (Fig. 2) were obtained from the *S. japonicum* Chinese cohort, comprising 66 individuals with HF and 18 controls living in the same region. Controls had not been exposed to infection with *S. japonicum* and were negative when tested by ELISA using (IgG) schistosome Ag. None of the cases and controls had active HBV infections and all cases had been treated with Praziquantel fewer than 10 times over the last 15 years. The mean age of cases and controls did not differ significantly and all individuals were >30 and <66 years old.

Cell Cultures and Cytokine Titration. PBMCs were separated from heparin-treated venous blood by Ficoll-Hypaque gradient sedimentation (400g for 30min at 18°C). PBMCs were washed and placed in cultures as described[24] and stimulated with 500 eggs/1ml well prepared as described.[24] Supernatants were collected at 72h and 144h and stored at −80°C. IL-22 and IL-17A concentrations were determined in the supernatant by ELISA (DuoSet kit, R&D, detection limits, 16 pg/ml).

Cellular Staining and Cytometry Analysis. PBMCs from Chinese patients were stained *ex vivo* with FITC-conjugated anti-CD4 and PE-Cy7-conjugated anti-CD3 (BD biosciences) antibodies. The labeled cells were treated for 5 hrs with 100 ng/ml phorbol-12-myristate-13-acetate (PMA) and 1 μg/ml ionomycin in the presence of monensin (Golgistop, BD biosciences). Cells were then fixed and permeabilized with BD Cytofix/Cytoperm according to the manufacturer's instructions and incubated with Alexa647-conjugated anti-IL-17 (eBiosciences) and PE-conjugated anti-IL-22 (R&D) antibodies. Isotype control antibodies were obtained from the corresponding manufacturers. Compensation settings were determined with Comp Beads (BD biosciences). Data were collected on a FACS Calibur cytometer with Cellquest software (BD biosciences) and analyzed with DIVA software (BD biosciences). Cells in the lymphocyte region were gated based on FSC and SSC proprieties and $CD3^+CD4^+$ cells or $CD3^-CD4^-$ cells in the lymphocyte gate were analyzed for the expression of IL-17 and IL-22.

RNA Extraction and Quantitative RT-PCR. Liver, spleen, Peyer patches and intestine biopsies were conserved in RNAlater (Ambion, Life Technologies) at −80°C. Tissue (20-30mg of tissue) homogenization was carried out with the Precellys-24 device (Bertin Technologies, Ozymee), with ceramic beads (1.4mm in diameter, CK14), in 400 μl of lysis buffer RLT plus (Qiagen) supplemented with 4μl β-mercapto-ethanol. RNA was recovered with the RNeasy plus following the manufacturer's instructions (Qiagen). RNA integrity was assessed with a 2100 Bioanalyzer (Agilent).

Total RNA (1μg), with a RIN > 7, was reverse-transcribed with the high Capacity cDNA Reverse

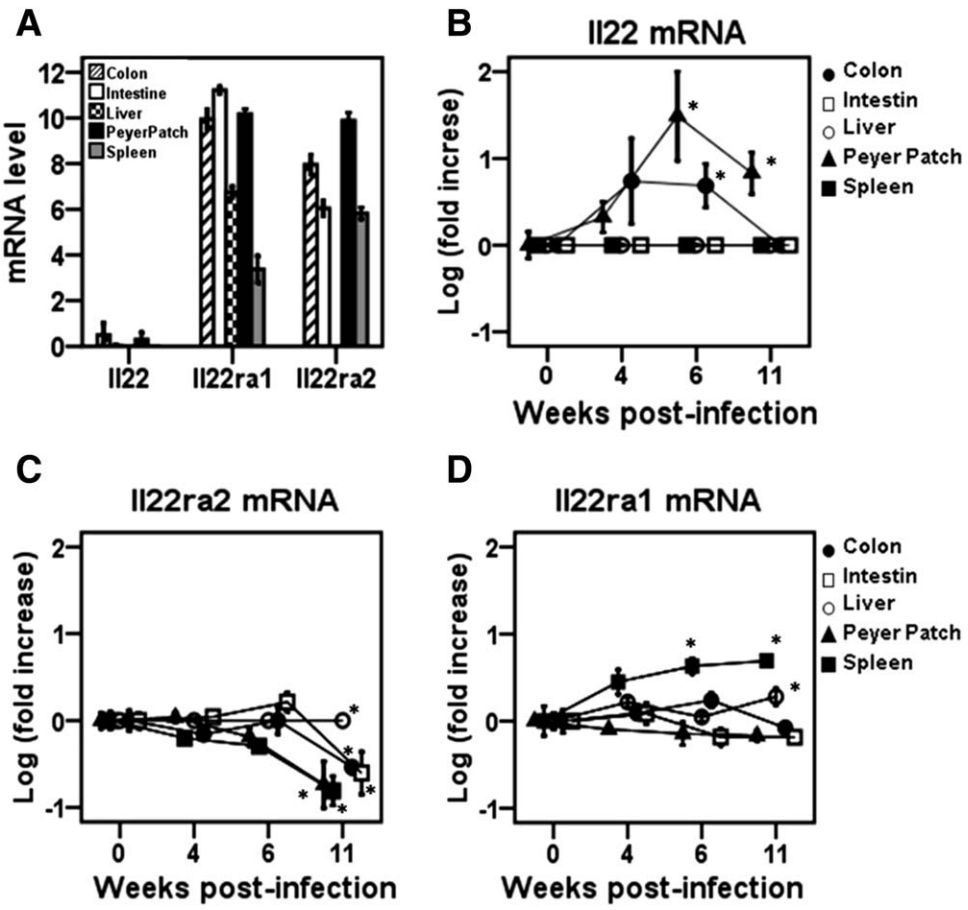

Fig. 1. Schistosome eggs stimulate the production of *IL22* mRNA and impair that of *IL22RA2* in infected mice (A-D). (A) Abundance of *Il22*, *Il22ra2*, and *Il22ra1* mRNA in colon, intestine, Peyer's patches, liver, and spleen of noninfected CBA/J mice (B-D) and mice infected with 30 *S. mansoni* cercariae. Transcript levels were evaluated at 4, 6, or 11 weeks postinfection. One representative experiment of two (5-6 mice per group) is shown. Data are the geometric mean (A) or the log (geometric mean) (B-D) of the fold change between infected and noninfected animals. Comparisons were performed by nonparametric analysis ($*P < 0.05$).

Transcription Kit (Applied Biosystems). Real-time quantitative PCR, from 20ng of cDNA, was performed with the ABI 7900HT Fast Real Time PCR System and TaqMan Universal PCR Master Mix (Applied Biosystems, Life Technologies). The TaqMan mouse gene expression assays: *Il22* (Mm00444241_m1), *Il22ra2* (Mm00617572_m1), *Il22ra1* (Mm00663697_m1), *Hprt1* (Mm00446968_m1) were from Applied Biosystems. Gene expression values were normalized to the value of the housekeeping gene *Hprt1* (hypoxanthine phosphoribosyltransferase).

Genotyping. DNA extraction and genotyping were performed as in Dessein et al.[23] Genotyped tag SNPs (Supporting Fig.2A) were selected from the 1000 Genomes Project and from the Hapmap database release #24, Genotyped SNPs had an R-square higher than 0.8 and minor allele frequency higher than 0.10. Primers are described in Supporting Table 2.

Statistical Analysis. Group comparisons performed on data from Fig. 1A-C were carried out with nonparametric Mann-Whitney tests with SPSS software. Linear regression analysis was performed to test for correlations between IL-22 concentrations, hepatic fibrosis and portal hypertension. CentF was divided into binary classes. NetF was divided in the same manner. Linear regression was performed with these binary variables[23] and with age, sex and exposure. Multivariate logistic regression (SPSS statistical software) was used to investigate possible associations of genotypes with advanced fibrosis or cirrhosis as described previously.[23]

Results

IL22, IL22RA2, and IL22RA1 Transcripts in Mice Infected With Schistosoma mansoni: Schistosome Eggs Stimulate Production of IL-22 in the Intestine. We measured Il22, Il22ra2, and Il22ra1 messenger RNA (mRNA) levels in the intestine, liver, and spleen of mice infected with Schistosoma mansoni to test whether schistosomes induce an IL-22/IL-22BP response (Fig. 1A-D). Il22 mRNA levels were low before infection (Fig. 1A); they started to increase in the colon and in Peyer patches, at 4 weeks when egg laying had just begun, and peaked at 6 weeks (Fig. 1B). Il22 mRNA was not detectable in liver or spleen. Il22ra2 mRNA levels were high in the intestine and spleen (but not in the liver) before infection (Fig. 1A) and were 5- to 10-fold lower after 11 weeks of infection (Fig. 1C). Thus, schistosome eggs promote

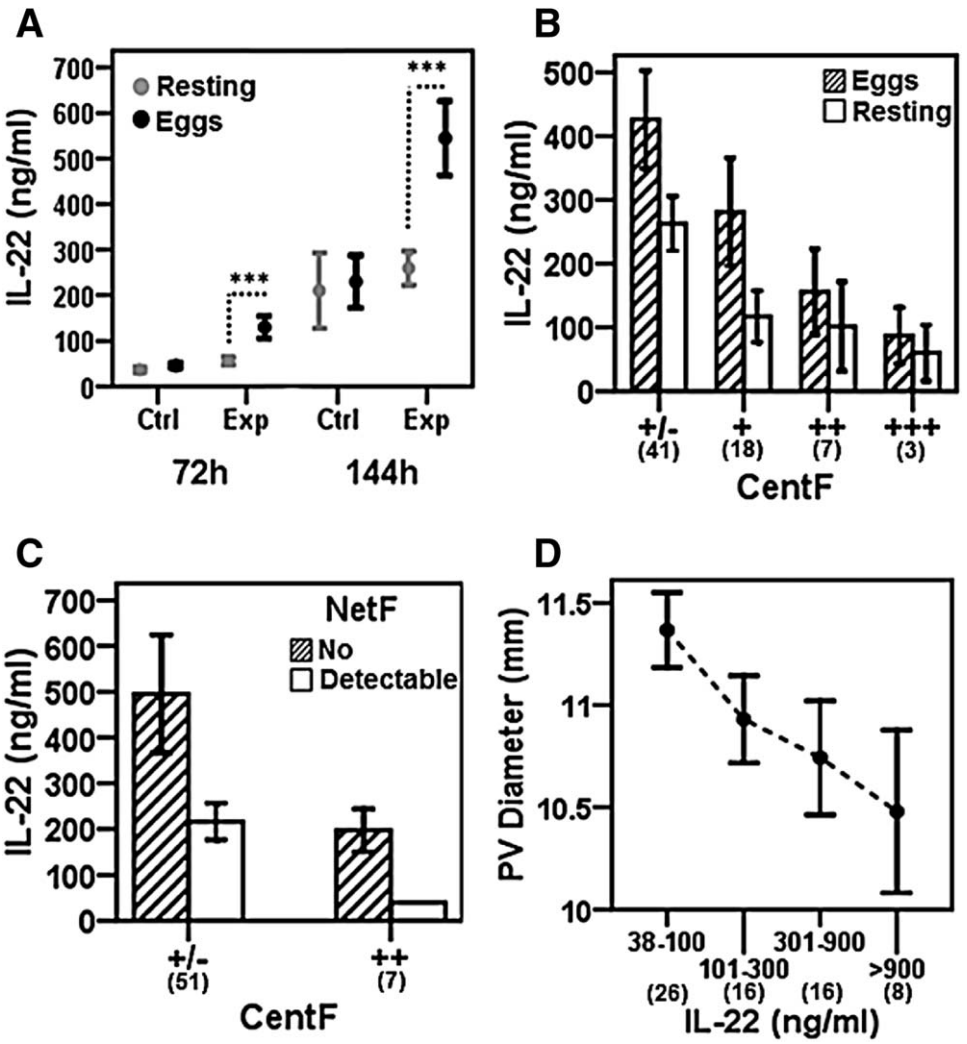

Fig. 2. IL-22 response in humans infected with schistosomes. IL-22 concentrations correlate with protection against HF and portal blood hypertension. (A) Concentration of IL-22 produced in egg-stimulated cultures of PBMCs from 66 Chinese fishermen and 18 controls. A total of 10^6 PBMCs were cultured for 72 or 144 hours with or without 500 schistosome eggs. (B) IL-22 concentrations in resting ($P = 0.005$) and egg-stimulated ($P = 0.04$) cultures at 144 hours are inversely correlated with CentF severity. Light CentF (CL^L) is (+/−) (n = 41), advanced CentF (CL^M) is (+) (n = 18), severe CentF (CL^H) is (++) (n = 7), and very severe CentF D+E+F is (+++) (n = 3). (C) Multivariate analysis shows that CentF and NetF are independently correlated with IL-22 levels in egg-stimulated cultures ($P = 0.009$ and $P = 0.004$, respectively). CentF was divided into CL^L, CL^M (n = 51), and CL^H, D+E+F (n = 7); NetF was divided into not detectable (n = 35) and detectable (n = 23). (D) PH (portal vein [PV] diameter) is inversely correlated with IL-22 produced in resting cultures at 144 hours ($P = 0.02$). IL-22 concentrations were divided into classes 11-100 (n = 26), 101-300 (n = 16), 301-900 (n = 16), and >900 ng/mL (n = 8). Bars show standard error of the mean.

production of Il22 mRNA and inhibit that of Il22ra2. Il22ra1 mRNA levels remained stable in the intestine and liver, and were high in the spleen after infection (Fig. 1D), indicating that these tissues can respond to IL-22.

Production of IL-22 by Blood Mononuclear Cells From Individuals With Chronic Schistosome Infection: IL-22 Is Produced by At Least Two Different Cell Populations. We evaluated the amount of IL-22 produced by peripheral blood mononuclear cells (PBMCs) from 66 Chinese fishermen infected with S. japonicum and 18 nonexposed controls (described in Materials and Methods). IL-22 was detectable in resting cultures of patient PBMCs at 72 and 144 hours of cell culture, and its production was stimulated by schistosome eggs ($P < 10^{-3}$; (Fig. 2A). IL-22 was detectable in cultures of control PBMCs only at 144 hours of culture, and its production was unaffected by eggs. IL-17A was detectable in cultures of PBMCs at 144 hours of culture, and its production was unaffected by eggs (data not shown). Flow cytometry analysis of patient PBMCs showed that both $CD3^+CD4^+$ and $CD3^-CD4^-$ cell populations produced IL-22, and neither cell population produced IL-17A (Supporting Fig. 1).[1,26,27]

IL-22 Produced by PBMCs From Individuals With Chronic Schistosomiasis Correlates With Protection Against HF and Portal Hypertension. HF in individuals infected with S. mansoni or S. japonicum manifests as central fibrosis (CentF) around the central vein, and in S. japonicum infections, a network fibrosis (NetF) pattern in the parenchyma is also present. In this study, CentF was graded as light (+/−), advanced (+), severe (++), and very severe (+++). Severe and very severe fibrosis were associated with portal blood hypertension and splenomegaly. We found that IL-22 concentrations in resting ($P = 0.005$) and in egg-stimulated ($P = 0.04$) cultures of PBMCs were inversely correlated with CentF (Fig. 2B) in PBMC donors infected with S. japonicum. Moreover, multilinear regression analysis showed that both severe and very severe CentF ($P = 0.01$) and NetF ($P = 0.004$) were independently associated with low IL-22 concentrations (Fig. 2C). Furthermore, patient portal vein diameter (an indicator of portal hypertension [PH]) was negatively correlated ($P = 0.02$) with IL-22 concentrations (Fig. 2D).

Polymorphisms in IL22RA2 Are Associated With Severe HF in Chinese Fishermen Exposed to S.japonicum; the Aggravating Genotypes Are Associated With an Increase of IL-22RA2 Transcripts. We determined whether polymorphisms in IL22RA2 affect the risk of severe HF to test further the hypothesis that high IL-22 production protects against schistosome-induced HF. IL22RA2 is located on Chr.6q23, which controls HF.[28] Mutations in connective tissue growth factor (CTGF) account for part of the control exerted by this locus.[23] IL22RA2, the gene encoding IL-22BP, is located in the same region and may also be involved in the control. To test this hypothesis, we genotyped single-nucleotide polymorphisms (SNPs) representative of the six major SNP correlation bins (TagSNP, $r^2 = 0.8$) in IL22RA2 (Supporting Fig. 2A; Supporting Table 1) in 327 Chinese fishermen (Table 1) with long exposure to S. japonicum infections. For this analysis, we have used a binary fibrosis phenotype that included, as cases, subjects with severe CentF or severe NetF and subjects with advanced CentF if they also had advanced or severe NetF. Control groups included individuals who exhibited no NetF and light CentF or less (see Materials and Methods). The genotypes AA, GG of SNP rs6570136 ($P = 0.003$; odds ratio [OR] = 2), and CC, TT of SNP rs7774663 ($P = 0.004$; OR = 2.1), both in bin I, and the genotypes CC, TT of SNP rs2064501 ($P = 0.01$; OR = 2), in bin VI were significantly associated with HF (Table 2; Fig. 3A,B). Multivariate analysis could not separate the effects of these SNPs owing to the high linkage disequilibrium (LD) between these SNPs (Fig. 3C).

Table 2. *IL22RA2* Genotypes Associated With Advanced Fibrosis in Chinese Fishermen Infected With *S. japonicum*

			Chinese Fishermen					
Analysis	SNP	Bins	Genotype	Controls	Cases	*P* Value	OR	95% CI
Univariate	6570136	I	AA, GG	45	62	0.003	2.07	1.5-5.4
	7774663	I	CC, TT	44	61	0.004	2.1	1.4-5.2
	7749054	II	GG, TT	46.2	54.8	0.12	1.45	0.9-2.3
	202563	III	AA	39.7	48.8	0.10	1.47	0.9-2.4
	276466	IV	AA	69.2	78.7	0.07	1.65	0.9-2.9
	11154915	V	TT, TC	25.6	34.6	0.10	1.5	0.9-2.6
	2064501	VI	CC, TT	53.6	66.4	0.01	2	1.2-3.3

Cases and controls are described in Material and Methods and in Table 1. Significant covariates in the logistic regression analysis were sex ($P = 10^{-3}$) and exposure ($P < 10^{-3}$). Bins represent correlation groups ($r^2 > 0.8$), and the aggravating genotype is shown.

Abbreviation: CI, confidence interval of OR.

Thus, rs6560136 and/or rs2064501, or other variants highly correlated with either of these SNPs, modulate susceptibility to HF in fishermen. We built a map of all SNPs correlated with either rs6560136 or rs2064501 in a region extending 5 Mb from the 3′ and 5′ end of *IL22RA2* to rule out the possibility that the casual variants may lie outside of *IL22RA2*. Supporting Fig 2B-D shows the various common SNPs (minor allele frequency >5%) in this region and their correlation with the SNPs of interest (y-axis). None of these SNPs outside *IL22RA2* were strongly correlated with rs6560136 or rs2064501 and could account for the association. Then, the causal SNPs must lie in *IL22RA2*.

Next, we evaluated whether rs6560136 and rs2064501 were associated with modulation of *IL22RA2* transcripts. We performed this analysis in healing skin tissue that highly expresses *IL22RA2*. We found a significant association of the genotypes GG, AA of rs6570136 ($P = 0.005$) and a suggestive association of the genotype TT of rs2064501 ($P = 0.065$) with the highest *IL22RA2* mRNA levels (Fig. 3D,E). This evaluation shows that the genotypes of rs6560136 that are associated with aggravation of fibrosis (Fig. 3A) are also associated with enhancement of IL22RA2 transcripts (Fig. 3D); they also suggest that the genotype TT of rs2064501, which is also associated with HF (Fig. 3B), also enhances IL-22RA2 transcripts (Fig. 3E).

Validation/Extension of the Associations Observed in Chinese Subjects to Sudanese and Brazilians Infected With S. mansoni. We sought to confirm the association between HF and SNPs in *IL22RA2* in Sudanese and Brazilian populations infected with *S.*

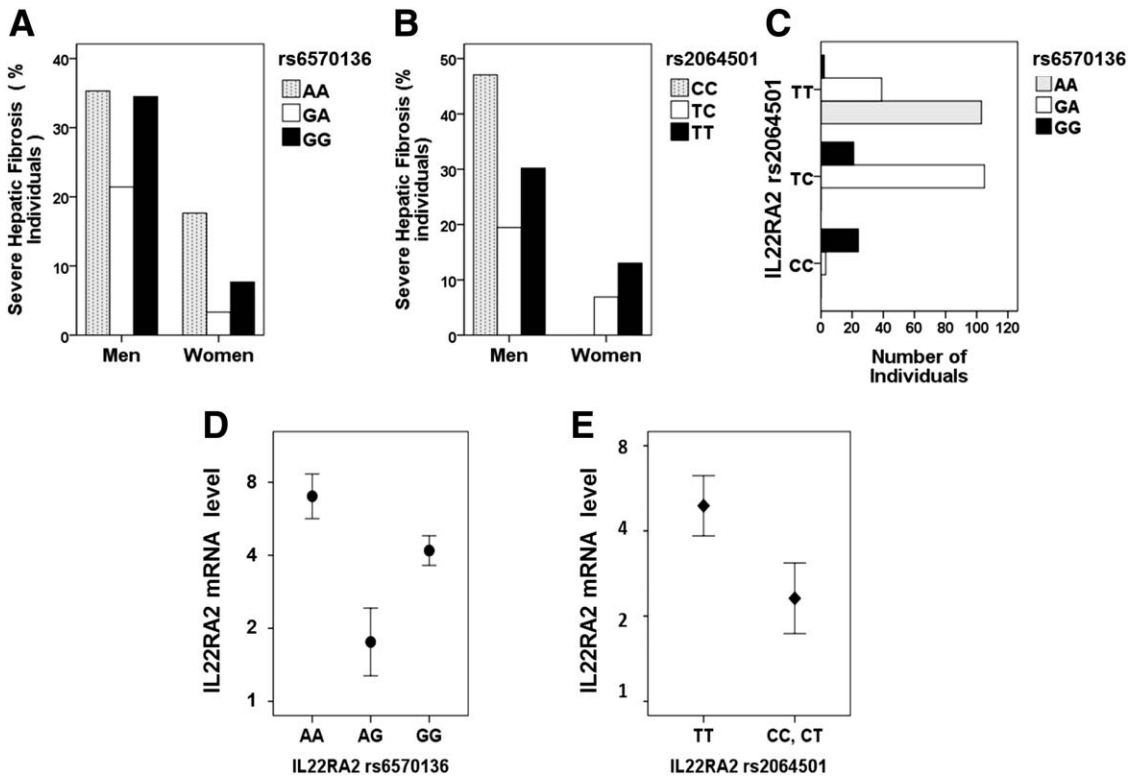

Fig. 3. *IL22RA2* genotypes that are associated with severe fibrosis in Chinese fishermen are associated with high *IL22RA2* mRNA levels in healing skin. (A and B) The genotypes AA and GG of SNP rs6570136 and the genotypes TT and CC of SNP rs2064501 are associated with susceptibility to severe HF. The sample of Chinese fisherman is described in Supporting Table 1, and data are also shown in Table 1. Data are represented for men and women separately ($P < 0.001$) and for fishermen who were exposed for more >20 years (longer exposure is not significantly associated with HF). (C) SNPs rs6570136 and rs2064501 are in strong linkage disequilibrium. (D) The genotypes AA and GG of SNP rs6570136 are associated ($P = 0.005$) with the highest *IL22RA2* mRNA levels in healing skin of 34 individuals. (E) Suggestive association of the genotype TT of SNP rs2064501 ($P = 0.065$) with the highest *IL22RA2* mRNA levels in the skin biopsies. The abundance of mRNA is expressed as arbitrary units (± standard error of the mean).

mansoni. We genotyped 201 Sudanese subjects (described in Table 1) and found that SNPs rs6570136 GG ($P = 0.03$; OR = 2.1), rs11154915 TT ($P = 0.04$; OR = 9.4), and rs2064501 CC, TT ($P = 0.03$; OR = 2.1) were associated with severe HF (Table 3). The best multivariate model included SNPs rs6570136 GG ($P = 0.0007$; OR = 8.2) and rs2064501 TT ($P = 0.02$; OR = 3.1).

We then genotyped 186 Brazilians (Brazil [1]; described in Table 1) and found that SNPs rs6570136 GG ($P = 0.001$; OR = 4.8), rs7774663 TT ($P = 0.02$; OR = 2.5), and rs779054 TT ($P = 0.02$; OR = 2.4) were associated with aggravation of HF (Table 3; Fig. 4A). No significant association was found between rs2064501 TT and HF in univariate analysis. Nevertheless, the best multivariate model included SNPs rs6570136 GG ($P = 0.003$; OR = 26) and rs2064501 TT, TC ($P = 0.03$; OR = 11). Thus, both rs6570136 and rs2064501 independently contribute to the association with severe HF, with rs6570146 GG and rs2064501 TT corresponding to the aggravating genotypes. In conclusion, variants of *IL22RA2* that are associated with a high abundance of *IL22RA2* transcripts are also associated with severe hepatic disease in three genetically different populations.

The Same Genetic Variants of IL22RA2 Are Associated With Susceptibility to Fibrosis and Cirrhosis in Individuals Infected With HCV. We evaluated whether these same polymorphisms affected fibrosis and cirrhosis in HCV-infected patients (n = 532, cohort Brazil [2]; Table 1) with different grades of HF (from F0 = no fibrosis to F3 = advanced fibrosis and F4 = cirrhosis). We found that the genotypes GG and AG of SNP rs6570136 ($P = 0.04$; OR = 1.6) and TT, CT of genotype rs2064501 ($P = 0.02$, OR = 2) and the genotype AA of the SNP rs202563 ($P = 0.07$; OR = 1.8) were associated with advanced fibrosis or cirrhosis (F3 and F4; n = 210) and were less prevalent in individuals with mild or no fibrosis (F0+F1+F2; n = 322; Table 4). Multivariate analysis demonstrated that rs6570136 GG, AG ($P = 0.007$; OR = 1.7) and rs2064501 TT, CT ($P = 0.004$; OR = 2.4) were

Table 3. *IL22RA2* Genotypes Associated With Advanced Fibrosis in Sudanese and Brazilian Farmers Infected with *S. mansoni*

			Sudanese Farmers						Brazilian Farmers					
Analysis	SNP (rs)	Bins	Genotype	Controls	Cases	*P* Value	OR	95% CI	Genotype	Controls	Cases	*P* value	OR	95% CI
Univariate	6570136	I	AA, GG	59.7	75.4	0.03	2.1	1.1-3.9	GG	13	35.6	0.001	4.8	2.1-11
	7774663	I	CC, TT	47	67.7	0.006	2.4	1.3-4.4	TT	25.7	44.2	0.02	2.5	1.1-5.2
	7749054	II	TT	34.2	47.7	0.07	1.7	0.95-3.1	TT	47.2	67.4	0.02	2.4	1.2-4.9
	202563	III	GG	22.8	35.5	0.06	1.9	0.98-3.5	GG, AG	74.2	86	0.13	2.1	0.8-5.5
	276466	IV				>0.3			AA	26.7	41.9	0.07	2	0.9-4.2
	11154915	V	TT	0.7	6.3	0.04	9.4	1.1-85	TT, TC	75.4	87	0.1	2.1	0.8-5.6
	2064501	VI	CC, TT	65.1	80	0.03	2.1	1.1-4.3	CC, CT	56.7	66.7	0.05	2.1	1.0-4.6
Multivariate	6570136		GG			0.0007	8.2	2.4-28.0	GG			0.003	26	3.1-222
	2064501		TT			0.02	3.1	1.2-8.1	TT, TC			0.03	11	1.2-102

The two principal schistosome strains that cause HF are *S. japonicum* in Asia and *S. mansoni* in Africa and South America. We investigated whether allelic variants of *IL-22RA2* also affect susceptibility to severe HF in an *S. mansoni*–endemic region in Sudan and Brazil. *IL22RA2* polymorphisms that were associated with HF in Chinese fishermen were genotyped in both samples. The phenotypes of cases and controls and the cohort size are described in Material and Methods and in Supporting Table 1. We first tested for associations between the SNPs and HF phenotypes separately (upper part of the table) and then tested the SNPs simultaneously (lower part). The aggravating genotype is shown. Age $P = 0.025$ was a covariate in the Sudanese multivariate analysis.

Abbreviation: CI, confidence interval of OR.

independently associated with severe fibrosis (F3+F4). The data obtained in comparing F3+F4 with F0+F1 are shown on Fig. 4B. These associations in HCV-induced HF in Brazilians are similar to the associations we have observed in schistosome-induced HF in Brazilians (Fig. 4A). The strong LD between the two associated SNPs (Fig. 4C) explains why the strength of the association is high when both SNPs are analyzed simultaneously.

We confirmed these results in an independent cohort (Brazil [3]; Table 1) of 149 (F0+F1 and F3+F4) patients infected with HCV, which showed that SNP rs6570136 GG (0.04) and rs2064501 TT (0.05) were independently associated with severe fibrosis (F3+F4).

Discussion

We evaluated production of IL-22 in a mouse model of schistosome infection to examine the role of IL-22/IL-22 BP in HF. In a previous study, IL-22 was not detected in the liver and spleen of schistosome-infected mice.[22] Although we confirmed this result, we also showed that the abundance of *IL22* transcripts is high in Peyer patches when schistosome eggs reach the mouse intestine. IL-22 is thought to protect epithelia against damage and mediates tissue repair.[5,12,13] IL-22 also stimulates production of antimicrobial peptides that limit the invasion of microbes across epithelial lesions.[2,6,7,11] Schistosome eggs perforate the intestinal wall; therefore, epithelial lesions caused by eggs, which allow the entry of bacteria and toxic bacterial products,

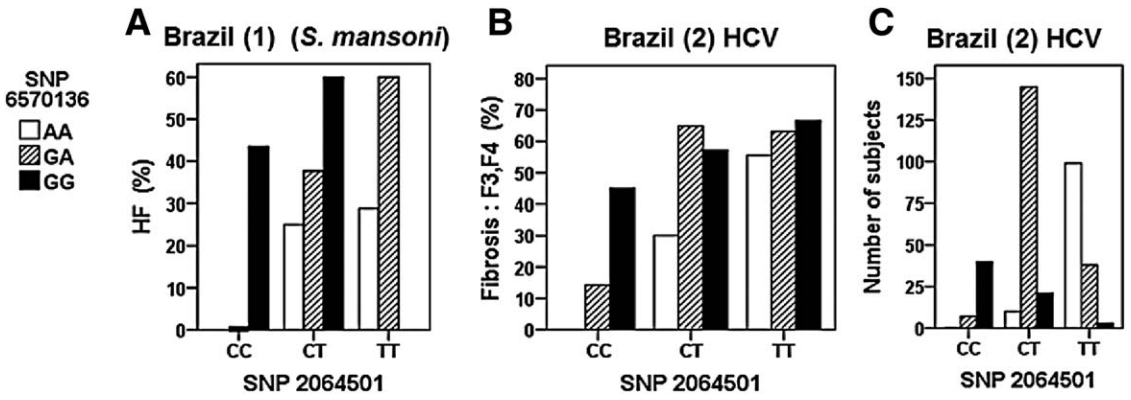

Fig. 4. The same alleles that are associated with susceptibility to severe HF in Brazilians infected with schistosomes are also associated with a high risk of fibrosis and cirrhosis in Brazilians infected with HCV. (A) The genotype GG of SNPs rs6570136 GG ($P = 0.003$) and the genotype TT, TC of SNP rs2064501 ($P = 0.03$) are independently associated with HF in Brazilians (Brazil [1]) infected with *S. mansoni*. (B) SNPs rs6570136 ($P = 0.007$) and rs2064501 ($P = 0.004$) are also independently associated with HF and cirrhosis in Brazilians (Brazil [2]) infected with HCV. The analysis compares F0+F1 with F3+F4, whereas data in Table 4 compare F0, F1, and F2 with F3 and F4. The SNP that shows the strongest association with HF is shown on the x-axis. (C) Distribution of rs6570136 and rs2064501 genotypes among Brazilians reveals strong LD between these two SNPs. The same genotype distribution was observed with schistosome- and with HCV-infected Brazilians inhabiting the same region of Brazil.

Table 4. *IL22RA2* SNP rs6570136 and SNP rs2064501 Are Also Associated With Fibrosis and Cirrhosis in Humans Infected With HCV

			HCV					
Analysis	SNP	Bins	Genotype	Controls	Cases	p	OR	95% CI
Univariate	6570136	I	GG,AG	65.2	72.2	0.04	1.6	1.0-2.2
	202563	III	AA	18.7	26	0.07	1.8	1.1-2.9
	2064501	VI	TT,CT	83.5	90.9	0.02	2	2.4
Multivariate	6570136	I	GG,AG			0.007	1.7	1.2-2.6
	2064501	VI	TT,CT			0.004	2.4	1.3-4.3

Co-variable : Age (45 yrs., $P = 0.001$), Alcohol ($P = 0.01$).

Severe (F3, F4) and mild (F0, F1, F2) fibrosis phenotypes and the cohort size are described in supporting Material and Methods and in Table 1. We first tested for associations between the SNPs and HF phenotypes separately (upper part of the table) and then tested the SNPs simultaneously by logistic regression analysis (lower part). Significant covariates ($P < 0.01$) were age (45 yrs., <46 yrs.) and alcohol intake. The aggravating genotypes are shown.

should induce a strong IL-22 response. Our failure to detect lL-22 in mouse liver is probably owing to a lack of sensitivity, because cells producing IL-22 in the liver are outnumbered by hepatocytes that do not produce it. We also found that the abundance of *IL-22RA2* transcripts in the intestine was reduced after the arrival of eggs. Thus, the balance between IL-22/IL-22BP is tipped in favor of IL-22 when eggs lodge in the liver and perforate the intestine. This indicates that the biological activity of IL-22 is high when hepatic and intestinal diseases begin to develop. A previous study found no evidence to suggest that IL-22 is involved in HF in schistosome-infected mice.[22] However, there are many differences between schistosomiasis in mice and humans. Liver disease in mice is evaluated within a few weeks of infection, whereas we examined hepatic disease in humans after more than 10 years of infection. Furthermore, the parasite load in mice is hundreds of times higher than in infected humans. For these reasons, we evaluated IL-22 in chronically infected individuals who have been living in an endemic region for their whole life. IL-22 was produced by PBMCs obtained from these individuals; IL-22 production was stimulated by schistosome eggs. Eggs had no effect on IL-22 production by PBMCs from local individuals who had no exposure to schistosomes. IL-17A production in these cultures was low and was not stimulated by eggs.[9,16,17] Our findings suggest that synergy between IL-22 and IL-17 does not occur in humans with long chronic infections, although we cannot fully exclude the possibility that IL-17 may be produced locally in particular tissues and not in the blood. Our data indicate that IL-22 in PBMC cultures was produced by at least two cell types: $CD3^+CD4^+$ and $CD3^-CD4^-$. This suggests that IL-22 is probably produced by Th22 cells[26,27] and natural killer–like innate lymphoid cells.[1] However, more work is required to characterize IL-22-producing cells in schistosome-infected patients. IL-22 was not produced by Th17 because little IL-17 was produced in cultures and IL-22-producing cells were IL-17 negative. There may be a high abundance of ILCs in the intestine because inflammation of the intestine strongly stimulates the multiplication of ILCs producing IL-22, as also indicated by our data in mice. We found that IL-22 production in cultures was positively correlated with protection against HF. Indeed, certain fishermen presented with no disease or only mild disease, although they have been exposed daily to infection for more than 20 years. This was owing to the effective control of HF and not inherent protection against infection. Portal vein diameter of the studied fishermen was inversely correlated with IL-22 levels. This measure is used to detect PH. Thus, high IL-22 levels are associated with protection against the most severe stages of HF.

To investigate further the link between IL-22/IL-22BP and HF, we performed a genetic analysis of *IL22RA2* to search for genetic variants associated with HF. We first studied *IL22RA2* because it is located in the 6q23 locus that exerts major control on HF in schistosome-infected populations.[28] Mutations in the *CTGF* gene, which is present at this same locus, contribute to this major genetic effect.[23] Nevertheless, *CTGF* cannot account for the entire effect of the locus. We obtained convincing associations between HF and two polymorphisms in *IL22RA2*. The observation that heterozygous individuals are better protected from HF than homozygous individuals of either genotype has been found in other infectious diseases. We believe that the strong LD between rs657136 and rs2064501 explains this effect. Our study in Sudan and China shows that the aggravating homozygous genotypes are rs657136 GG and rs2064501 TT. The protective homozygous genotype of one SNP is almost always associated with the aggravating homozygous genotype of the other SNP owing to the strong LD between these SNPs. This association between

homozygous genotypes with opposite effects neutralizes the effects of the protective genotypes and gives rise to a genotype that is associated with disease aggravation. The association of variants in *IL22RA2* with HF directly implicates IL-22BP in HF. Homozygous genotypes, which are associated with susceptibility to severe HF, are also associated with high levels of *IL22RA2* transcripts, strongly suggesting that IL-22BP aggravates HF. This finding is consistent with the association we observed between IL-22 and protection against both HF and PH.

The underlying mechanisms that lead to HF in schistosome and HCV infections are similar in many aspects. However, in HCV infections, liver fibrosis, and viral hepatotoxicity are associated with a vigorous multiplication of hepatocytes, which is not observed in schistosome infections. IL-22 promotes liver cell regeneration by increasing cell proliferation and hepatocyte migration.[29] However, such proliferation contributes to the regeneration nodules that greatly augment loss of liver architecture and organization and impairment of liver function in cirrhosis. Hepatocyte proliferation may be aggravated by IL-22, which stimulates tissue regeneration and inhibits apoptosis[10,30]; furthermore, uncontrolled IL-22 activity may promote development of hepatocarcinoma.[14] For these reasons, we investigated these same polymorphisms in the context of HCV-induced cirrhosis. We showed that genetic variants of *IL22RA2* that are associated with susceptibility to severe HF in schistosomiasis are also associated with HCV-induced cirrhosis, indicating that IL-22/IL-22BP exerts hepatoprotective effects in both HCV and schistosome infections.

Several biological effects may account for the protective action of IL-22 against schistosome-induced HF. IL-22 may stimulate production of anti-inflammatory molecules,[31,32] promote liver repair by limiting apoptosis, or stimulating mitosis, cell migration,[20,31] and progenitor cell growth.[33] IL-22 may also promote stellate cell senescence.[34] Regulation of IL-22 by IL-22BP may also limit entry of profibrogenic bacterial products, such as lipopolysaccharide (LPS), by stimulating antibacterial innate immunity,[4,35] reducing intestinal inflammation,[36,37] and promoting healing of the intestinal epithelium,[12,38] which is perforated by thousands of schistosome eggs. LPS is known to stimulate hepatic fibrogenesis through direct effects on hepatic stellate cells, which express the Toll-like receptor 4 receptor. Finally, IL-22 may stimulate the liver to produce LPS-binding protein.[39] Intestinal damage does not typically occur during HCV infections; therefore, the protective effects of IL-22 are likely to result from a direct protective action on the liver[10,40] and probably involve tissue repair and regeneration[21] and stellate cell senescence,[34] which was shown to be crucial for limiting HF. Hepatic tissue repair is probably more critical in HCV infections than in schistosome infections because HCV is very cytotoxic for hepatocytes, whereas schistosomes are not because eggs are trapped in the liver sinusoids and toxic substances are prevented from diffusing by sequestration in the granuloma.

In conclusion, we show that IL-22 is associated with protection against liver fibrosis in human schistosomiasis, and mutations that promote *IL-22BP* expression, the physiological inhibitor of IL-22, aggravate fibrosis and cirrhosis in both schistosome and HCV infections. These results strongly suggest that IL-22 protects against HF and cirrhosis. This is also the first direct evidence that IL-22BP plays a significant regulatory role in human inflammatory diseases. IL-22 and IL-22BP do not act on hematopoietic cells; therefore, pharmacological intervention against these molecules should have fewer side effects than treatments that target classical cytokines such as tumor necrosis factor. Thus, IL-22 and IL-22BP may be good therapeutic targets[3] in the prevention and treatment of fibrosis and cirrhosis.

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